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TITLE OF INVENTION: USE OF GLYCURONIC POLYSACCHARIDES AND OLIGOSACCHARIDES AS PHYTOSANITARY PRODUCTS AND/OR FERTILISER

APPLICANT(S) FOR DO/EO/US: Yvette LIENART, Alain HEYRAUD and Olivier SEVENOU

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau ***--in French language***).
   b. [ ] has been transmitted by the International Bureau. (see attached copy of PCT/IB/308)
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Item 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: INTERNATIONAL PRELIMINARY EXAMINATION REPORT (PCT/IPEA/409), INTERNATIONAL SEARCH REPORT (PCTISA/210), APPLICATION DATA SHEET

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Yvette LIENART et al. Box Non-fee Amendment

Serial No. (unknown) GROUP

Filed herewith Examiner

USE OF GLYCURONIC POLYSACCHARIDES AND OLIGOSACCHARIDES AS PHYTOSANITARY PRODUCTS AND/OR FERTILISER

PRELIMINARY AMENDMENT

Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to the first Official Action and calculation of the filing fee, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend claims 3-5, 9 and 11 as follows:

--3.(Amended) Use according to claim 1 of 1,4 D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

4.(Amended) Use according to claim 1 of 1,4 b-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$, the weight percentage of $COCH_3$ preferably being between 0 and 30.5.

5.(Amended) Use according to claim 1 of b(1-4) chain glycuronic oligosaccharides, such as the oligo 1,4 b-D-glucuronans, the oligo 1,4 b-D-mannuronans, and the oligo 1,4 b-D-guluronans, whose DP is less than 30, and preferably between 2 and 15.

9.(Amended) Use according to claim 7, of oligo 1,4 b-D-glucuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 b-D-glucanase, and the enzyme 1,4 b-D-glucanase, within the framework of control of one or more stages of plant development, such as the control of fruit maturation, abscission, growth of the pistil or maturation of the anthers.

11.(Amended) Use according to claim 7, of oligo 1,4 b-D-mannuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme xyloglucan endotransglycolase within the framework of the control of organization of cell walls during expansion of the tissues and/or to reinforce the plant cell walls and adapt them to environmental stimuli.--

REMARKS

Claims 3-5, 9 and 11 were amended to correct multiple dependency. Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned **"VERSION WITH MARKINGS TO SHOW CHANGES MADE"**.

Respectfully submitted,

YOUNG & THOMPSON

By _____

Thomas W. Perkins
Attorney for Applicant
Customer No. 000466
Registration No. 33,027
745 South 23rd Street
Arlington, VA 22202
703/ 521-2297

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"VERSION WITH MARKINGS TO SHOW CHANGES MADE"

Claims 3-5, 9 and 11 have been amended as follows:

3. (Amended) Use according to claim 1 ~~or 2~~ of 1,4 D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

4. (Amended) Use according to claim 1 ~~or 2~~ of 1,4 b-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$, the weight percentage of $COCH_3$ preferably being between 0 and 30.5.

5. (Amended) Use according to claim 1 ~~or 2~~ of b(1-4) chain glycuronic oligosaccharides, such as the oligo 1,4 b-D-glucuronans, the oligo 1,4 b-D-mannuronans, and the oligo 1,4 b-D-guluronans, whose DP is less than 30, and preferably between 2 and 15.

9. (Amended) Use according to claim 7 ~~or 8~~, of oligo 1,4 b-D-glucuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 b-D-glucanase, and the enzyme 1,4 b-D-glucanase, within the framework of control of one or more stages of plant development, such as the control of fruit maturation, abscission, growth of the pistil or maturation of the anthers.

11. (Amended) Use according to claim 7 ~~or 8~~, of oligo 1,4 b-D-mannuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme xyloglucan endotransglycolase within the framework of the control of organization of cell walls during expansion of the tissues and/or to reinforce the plant cell walls and adapt them to environmental stimuli

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USE OF GLYCURONIC POLYSACCHARIDES AND OLIGOSACCHARIDES AS PHYTOSANITARY PRODUCTS AND/OR FERTILIZERS

The present invention concerns the use of 1,4 β-D-glucuronan polymers and derived glycuronic oligosaccarides as phytosanitary products and/or fertilizers.

The enzyme 1,3-β-D-glucanase is a marker of defence mechanisms in plants. During reactions caused by hypersensitivity to a pathogen (bacteria, fungi, viruses), the plant reacts by inducing the synthesis of specific proteins named "PR-proteins" (Sintzi A. et al. (1993) Biochimie, 75, 687-706). These proteins, linked with pathogenesis, together with other molecules (such as salicylic acid) contribute to the development of resistance to the pathogen. Depending on their biochemical properties, and their physiological function, these proteins are listed in several groups. They share the following characteristics: low molecular weight, composition most often monomeric, resistance to proteolysis, stability in an acid medium or in extreme temperatures, their association with plasmic or endoplasmic membranes, and their parietal location. Amongst these PR proteins is Group 2, composed of 1,3 β-D-glucanase enzymes, which recognize 1,3 β-D-glucane chains as substrates. The role of these proteins in the defence of the plant is based on their capacity to lyse the walls of pathogens which are rich in 1,3 β-D-glucanes (Boller T. (1993) In Mechanisms of Plant Defenses Responses. Fritig B. Legrand M. eds. Kluwer. Acadamic Publishers Dordrecht, 391-400).

However, this enzyme activity is not only involved in the defence of plants. In fact it can be regulated by phytohormones and it can be induced at certain stages of development of the plant. On this basis the enzyme 1,3-β-D-glucanase is a marker of growth and/or cell differentiation in plants.

This enzyme, like a number of PR-proteins (protease inhibitors, chitinases, proteins regulating the expression of genes encoding osmotin) are associated with growth and/or cell differentiation or with processes of adaptation to the environment. Some of these proteins are recognized by antibodies directed against 1,3-β-D-glucanases isolated from tobacco contaminated with tobacco mosaic (Kauffmann et al. (1990) Plant Mol. Biol. 14(3): 381-90).

Activities of 1,3-β-D-glucanase or genes encoding these proteins are induced in the course of germination, and development of the flower buds and fruits (del Campillo E., Lewis L.N. (1992) Plant Physiology 99, 1015-1020; Neale et al. (1990) Plant Cell 2, 7, 673-684). In particular, these responses develop in the tissues by means of catabolic modifications (endosperm, pollen tubes, stem abscission zones, peduncles etc.), or during the period of mitotic division (case of anthers, stigmas, stems). They are dependent on hormones (auxins, cytokinins in general, absisic acid in particular) and molecules such as ethylene, controlling the maturation of fruits, or salicylic acid, controlling flowering, are also inducers. Finally, 1,3 β-D-glucanase enzymes have been recorded for functions of adaptation of the plant to cold and to raised ozone levels (Hincha et al. (1997) Plant physiology 114, 1077-1083).

The enzyme 1,4 β-D-glucanase is a marker of growth and/or cell differentiation in plants. This enzyme recognizes as substrate, linear chains of glucanes linked at β (1,4). It can hydrolyse cellulose, β glucanes (1,4) (1,6), and xyloglucan. Thus, it plays a part in the ultrastructural modifications of the walls of plant cells during the growth process. Its induction and/or that of the specific genes is revealed during processes involving the lysis of plant cell walls, rupture of the anthers, abscission zones of fruits and flowers (Hayaschi T., Oshimi C. (1994) Plant Cell Physiology 35(3), 419-424; Brummel D.A. et al. (1997) Plant Biol. Mol. 33, 1, 97-195). It is controlled by ethylene, by hormones such as abscisic acid or auxin.

Xyloglucan endotransglycolase activity induces the modification of the xyloglucans of plant cell walls in response to environmental stimuli such as mechanical pressure, wind, darkness, and thermal shocks (Xu et al. (1996) Plant J. 9(6), 879-89; Antosiewicz et al. (1997), 115(4), 1319-28).

The present invention arises from the inventors' demonstration of the fact that 1,4 β-D-glucuronans and glycuronic oligosaccharides derived from the latter have activities amplifying the enzyme 1,3 β-D-glucanase and/or the enzyme 1,4 β-D-glucanase, and/or the enzyme xyloglucan endotransglycolase, and, on this basis, are described as "eliciting" compounds, which can be used within the framework of phytosanitary uses or fertilization.

1,4 β-D-glucuronan polymers have already been described in the French patent FR-B-2 688 222 of 3 March 1992, relating to areas of use totally different from those mentioned above for the present invention, i.e. in the domains of food, pharmaceuticals,

human or veterinary therapy, cosmetics or water purification, in particular as a gelling, thickening, hydrating, stabilizing, chelating or flocculating agent, as well as in the preparation of oligosaccharides.

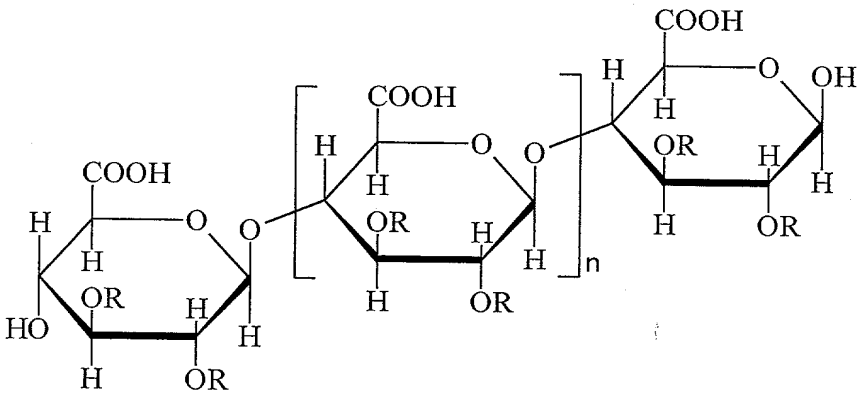
The present invention has the aim of providing compounds which can be used as "elicitors" included in the composition of fertilizers (manure, biological fertilizers or biofertilizers) and phytosanitary products.

One of the aims of the present invention is to provide new biofertilizers which can be used in particular as stimulants of nutrition in addition to or as a replacement for commercial products based on potash and nitrates which are toxic to the environment, and/or as regulators of one or more stages of development of the plants.

Another aim of the present invention is to provide new phytosanitary products which can be used in particular as activators of defence and resistance reactions against biotic or abiotic stresses in addition to or as a replacement for pesticides which are toxic to the environment.

The present invention has as its subject the use of compounds chosen from:

– 1,4 β-D-glucuronan polymers of formula (I) below:



in which n is an integer which may be as great as approximately 2500, advantageously n is between approximately 300 and approximately 2500, and R represents H or $COCH_3$.

– and/or the β(1-4) chain glycuronic oligosaccharides derived from polymers of formula (I), and of which the number of saccharidic units is less than approximately 30, and preferably between 2 and 15,

– and/or the esters and/or ethers corresponding to polymers of formula (I) or to the above mentioned oligosaccharidic derivatives,

* as phytosanitary products within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase,
* and/or as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and/or the enzyme 1,4 β-D-glucanase, and/or xyloglucan endotransglycolase.

Of the plants which can be treated within the framework of the present invention, the following may be cited: vines, fruit trees, cereals and market garden produce, or any other plant of economic interest.

The invention has more particularly as its subject the above mentioned use of compounds chosen from those cited above, as phytosanitary products within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, such as the protection of plants against pathogens or predators, notably against bacteria, viruses, fungi, insects, nematodes, or the adaptation of plants to an abiotic stress, in particular adaptation to cold, or to raised ozone levels.

The invention has more particularly as its subject the use, as phytosanitary products, of 1,4 β-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

The invention also has more particularly as its subject the use, as phytosanitary products, of 1,4 β-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, R represents H or $COCH_3$, the weight percentage of $COCH_3$ preferably being between 0 and 30.5.

The invention also concerns the use, as phytosanitary products, of β(1-4) chain glycuronic oligosaccharides, such as the oligo 1,4 β-D- glucuronans, the oligo 1,4 β-D-mannuronans, and the oligo 1,4 β-D-guluronans, whose DP (degree of polymerization) is less than 30, and preferably between 2 and 15.

In the following, the expression "oligosaccharides with a degree of polymerization x (DPx)" shall be understood as meaning oligosaccharides made up of the same number x of saccharidic units, and the expression "oligosaccharides with an average degree of polymerization x (average DP x)" shall be understood as meaning oligosaccharides made up of a variable number of saccharidic units, whose average corresponds to x.

Glycuronic oligosaccharide derivatives preferred as phytosanitary products are chosen from the following:

- the oligo 1,4 β-D-glucuronans of DP8, and of average DP 8
- the oligo 1,4 β-D-mannuronan of DP4,
- the oligo 1,4 β-D-guluronan of DP4.

The invention also has as its subject a process for the treatment of plants with 1,4 β-D-glucuronan polymers and/or glycuronic oligosaccharides as defined above, with a view to obtaining plants resistant to the above mentioned pathogens, or adapted to an abiotic stress, notably the cold, or raised ozone levels.

The invention also concerns the use of compounds chosen from those cited above, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and/or the enzyme 1,4 β-D-glucanase and/or the enzyme xyloglucan endotransglycolase.

The invention has more particularly as its subject the above mentioned use of oligo 1,4 β-D-glucuronans, whose DP is below 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and/or the enzyme 1,4 β-D-glucanase, notably within the framework of control of one or more stages of plant development, such as the control of fruit maturation, abscission, growth of the pistil or maturation of the anthers.

The invention has still more particularly as its subject the above mentioned use of oligo 1,4 β-D-glucuronans of DP8 and average DP 8, as biofertilizers.

The invention has more particularly as its subject the above mentioned use of oligo 1,4 β-D-mannuronans, whose DP is below 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme xyloglucan endotransglycolase, notably within the framework of control of the organization of cell walls during expansion of the tissues (vascular zones or parenchyma) of certain organs such as hypocotyls, cotyledons, leaves, pollen tubes, and fruits, and to reinforce the plant cell walls and adapt them to environmental stimuli such as wind, thermal or water shock, or mechanical pressure.

The invention also has as its subject a process for the treatment of plants with glycuronic oligosaccharides as defined above, with a view to obtaining plants in which one or more stages of development, such as fruit maturation, abscission, growth of the pistil or maturation of the anthers, are controlled over time.

The invention also has as its subject a process for treatment of plants with glycuronic oligosaccharides as defined above, with a view to obtaining plants, in which the organization of cell walls during expansion of the tissues is controlled, and in which the plant cell walls are reinforced to adapt them to environmental stimuli such as wind, thermal or water shock, or mechanical pressure.

The invention also concerns phytosanitary products and/or biofertilizers characterized in that they include at least one compound chosen from:

– 1,4 β-D-glucuronan polymers of formula (I) mentioned above, in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$,

– and/or β(1-4) chain glycuronic oligosaccharides derived from polymers of formula (I), and of which the number of saccharidic units is less than approximately 30,

– and/or the esters and/or ethers corresponding to polymers of formula (I) or to the above mentioned oligosaccharidic derivatives.

The invention has more particularly as its subject phytosanitary products comprising at least one 1,4 β-D-glucuronan polymer of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

The invention also concerns phytosanitary products comprising at least one 1,4 β-D-glucuronan polymer of formula (I) in which n is an integer between approximately 300 and approximately 2500, R represents H or $COCH_3$, the percentage of $COCH_3$ by weight being preferably between 0 and 30.5.

The invention also has as its subject phytosanitary products, comprising at least one β(1-4) chain glycuronic oligosaccharide, such as the oligo 1,4 β-D- glucuronans, the oligo 1,4 β-D-mannuronans, and the oligo 1,4 β-D-guluronans, whose DP is less than 30, and preferably between 2 and 15.

The invention has more particularly as its subject, phytosanitary products comprising at least one glycuronic oligosaccharide derivative chosen from the following:

– the oligo 1,4 β-D-glucuronans of DP8, and of average DP 8

– the oligo 1,4 β-D-mannuronan of DP4,

– the oligo 1,4 β-D-guluronan of DP4.

The invention concerns more particularly biofertilizers comprising at least one oligo 1,4 β-D-glucuronan whose DP is below approximately 30, and preferably between

2 and 15, and, preferably, biofertilizers comprising oligo 1,4 β-D-glucuronans of DP8, and of average DP 8.

The invention concerns still more particularly biofertilizers comprising at least one oligo 1,4 β-D-mannuronan whose DP is below approximately 30, and preferably between 2 and 15, and, preferably, biofertilizers comprising the oligo 1,4 β-D-mannuronan of DP4.

The invention will be further described by means of the following detailed description of the preparation of 1,4 β-D-glucuronan polymers and glycuronic oligosaccharides derived according to the invention, as well as the demonstration of their properties of amplifying the enzyme 1,3 β-D-glucanase and/or the enzyme 1,4 β-D-glucanase.

A) Preparation of uronic polymers and/or their oligosaccharides

1,4 β-D-glucuronan polymers are obtained using fermentation processes of strains of bacteria isolated from the rhizosphere (*Rhyzobium, Sinorhyzobium, Agrobacterium, Pseudonomas, Burkolderia etc.*) which may or may not be modified by chemical mutations and/or genetic manipulations.

1,4 β-D-glucuronan polymers can thus be obtained by selective oxidation of cellulose according to the processes described in various articles (Painter T.J., 1977, Preparation and periodate oxidation of C-6-oxycellulose: conformational interpretation of hamlacetal stability. Carbohy. Res. 55, 95-103; Chang P.S. and Robyt J.F., Oxidation of primary alcohol groups of naturally occurring polysaccharides with 2, 2, 6, 6-tetramethyl-1-piperidine oxoammonium ion. J. Carbohydr. Chem., 15, 819-830; Isogai, A. and Kato, Y., 1998, Preparation of polyuronic acid from cellulose by TEMPO-mediated oxidation, Cellulose, 5, 153-164).

If necessary, the polymers thus obtained are modified and/or degraded by chemical and/or enzymatic means, during fermentation or by post-fermentation treatments.

- 1,4 β-D-glucuronan polymer

By way of illustration, 1,4 β-D-glucuronan polymer is obtained by fermentation of a mutated strain of *Rhizobium meliloti*, according to the protocol described in the French patent FR-B-2 688 222 of 3 March 1992.

- 1,4 β-D-glucuronan oligomer of average DP 8

The previous native polymer, i.e. comprising at least 50% acetylated glucuronic units at C2 and at C3, is subjected to enzymatic hydrolysis. The enzyme is a glucuronate lyase of various origins, notably extracted from the pancreas of ormers, or of fungal origin (Dantas L. et al., Carbohydr. Res., 265 (1994) 303-310) or a glucuronate lyase present in the culture medium of bacteria such as strains of Rhizobiaceae (Michaud P., et al., Int. J. Hiol. Macromol. 21 (1997) 3-9). The mixture of oligosaccharides thus obtained is deacylated by a basic process (NaOH 0.1M), then fractionated on the basis of the degree of polymerization (DP) by gel-permeation chromatography using a BioGel P6 column (Dantas L. et al., mentioned above).

- 1,4 β-D-mannuronan and 1,4 β-D-guluronan oligomers of DP 4.

The starting polymer is an alginate, linear copolymer of mannuronic (M) and guluronic (G) acids, whose M/G ratio and mode of arrangement depend on the origin. The alginate is chosen on the basis of the type of oligomers to be prepared. Hydrolysis is carried out by enzymatic means: an alginate-lyase of ormer origin for the oligo-1,4 β-D-mannuronans (Heyraud A. et al., Carbohydr. Res., 291 (1996) 115-126), an alginate-lyase of bacterial origin for the oligo-1,4 β-D-guluronans (Patent FR 97 03218 of 11 March 1997). The different oligosaccharides, separated according to DP by gel-permeation chromatography, are then purified depending on their structure by ionic chromatography in high-pressure liquid chromatography according to the process described in the article by Heyraud et al., mentioned above.

B) 1,3 β-D-glucanase response induced in the protoplasts of *Rubus.*

Experimental conditions: (1) preparation of protoplasts from cell suspensions of *Rubus fruticosus L.*; (2) incubation, or not, of n samples of 2.10^6 protoplasts in the presence of "elicitors" (1,4 β-D-glucuronan polymers and 1,4 β-D-galacturonan (400 µg/L) polymers, 1,4 β-D-glucuronan oligomers of average DP 8, 1,4 β-D-mannuronan oligomers of DP 4, and 1,4 β-D-guluronan oligomers of DP 4 (50 nM)); (3) after 20

minutes, the protoplasts whether may be treated or not, are subjected to enzymatic extraction. 2 µg of proteins are used per enzymatic test, and per incubation period. The viability of the protoplasts is maintained at 95% for an experimental period of 6 hours; the Evans blue viability test used verifies the integrity of the plasmalemma.

Methodology: measurement of activity (1,3 β-D-glucanase) is based on the colorimetric dosage (ferricyan test) of the substrate-reducing units (reduced hexamer from laminarin) released during hydrolysis. Based on the kinetics developed, curves are traced whose equations make it possible to calculate the speed of the enzymatic reaction. 2 kinetics, at least, are developed per sample, and per experimental "set". In general, at least 8 kinematics from samples of 2 independent "sets" are developed.

Results: the enzymatic activation elicited in the protoplasts is expressed as a % of the activity in the controls. The results are summarized in Table 1.

Table 1

"elicitor"	activity (% control)
a	145
b	128
c	100
d	122
e	146

Table 1: Comparative analysis of the responses (1,3-β-D-glucanase) induced by the "elicitor" (1,4 β-D-glucuronan polymer (400 µg/L) (a), oligo 1,4-β-D-glucuronan of average DP 8 (50 nM) (b), 1,4-β-D-galacturonan (400 µg/L) (c), oligo 1,4-β-D-mannuronan of DP 4 (50 nM) (d), oligo 1,4-β-D-guluronan of DP 4 (50 nM) (e).

Electrophoretic analysis by SDS-PAGE of the proteins making up the enzymatic extracts has been carried out. The marking of proteins on prints by a serum recognizing 1,3-β-D-glucanases isolated from tobacco contaminated with tobacco mosaic (Ori et al. (1990) EMBO J., 9(11), 3429-36) confirms the presence of PR proteins.

The 1,4-β-D-glucuronan oligomer of average DP 8 and the polyglucuronan, used at a nanomolar concentration, in 20 minutes amplify by a factor of 1.5 and 1.3

respectively the 1,3-β-D-glucanase activity in plant protoplasts. Amongst the other products tested, oligo 1,4-β-D-guluronan of DP4 was the most effective.

C) 1,4 β-D-glucanase response induced in protoplasts of *Rubus*

Experimental elicitation conditions: identical to those reported above.

Methodology: measurement of activity (1,4 β-D-glucanase) is based on the colorimetric dosage (ferricyan test) described above of the substrate-reducing units (reduced cellopentaose) released during hydrolysis.

Results: The results are shown in Table 2.

Table 2

"elicitor"	activity (% control)
a	100
b	120
c	100
d	-
e	101

Table 2: Comparative analysis of the responses (1,4 β-D-glucanase) induced by the "elicitor" (1,4 β-D-glucuronan polymer (400 µg/L) (a), oligo 1,4 β-D-glucuronan of average DP 8 (50 nM) (b), 1,4 β-D-galacturonan polymer (400 µg/L) (c), oligo 1,4 β-D-mannuronan of DP 4 (50 nM) (d), oligo 1,4 β-D-guluronan of DP 4 (50 nM) (e).

The 1,4-β-D-glucuronan oligomer of average DP 8 used at a nanomolar concentration, in 20 minutes amplifies by a factor of 1.2 the 1,4 β-D-glucanase activity in plant protoplasts.

D) Xyloglucan endotransglycolase response induced in protoplasts of *Rubus*

Experimental elicitation conditions. 2.10^6 protoplasts in 1 ml Tris-HCl buffer (pH 4.8) are incubated in the presence, or not, of an elicitor (50 nM) or of a hormone (50nM): mannuronan oligomer of DP4 or glucuronan oligomer of DP8 or gibberellin

GA_3. After 20, 40, 60, 100 and 120 minutes of interaction, the protoplasts are recovered by centrifugation, then subjected to enzymatic extraction.

Methodology: measurement of XET activity is carried out in the wells of microtitration plates in 4 stages. Stage 1: immobilization of the acceptor, i.e. the neoglycoprotein XXLG ≈ BSA. Stage 2: introduction of the reaction medium (XET enzymatic extraction (equivalent to 1 µg of proteins) substrate marked DIG, i.e. XG ≈ DIG in the Tris-HCl buffer, pH = 7, 25 mM). Stage 3: immunomarking according to the anti-DIG marked peroxydase sequence, anti-peroxydase marked peroxydase. Stage 4: dosage of the peroxydase activity in a citrate-phosphate buffer (50nM, pH 5.5).

The peroxydase activity is measured at 492 nm. At least 3 curves of peroxydase activity are traced per experimental condition, and the experiments are carried out using 3 protoplast suspensions. The XET activity is measured by the slope (ΔA 492) of the curve deduced by linear regression from 9 peroxydase kinetics curves.

Abbreviations:

DIG: digoxygenin

XET: xyloglucan endotransglycolase - XG: xyloglucan polymer - XXLG: non-fucosylated xyloglucan oligomer.

Results: The results are indicated in Figure 1.

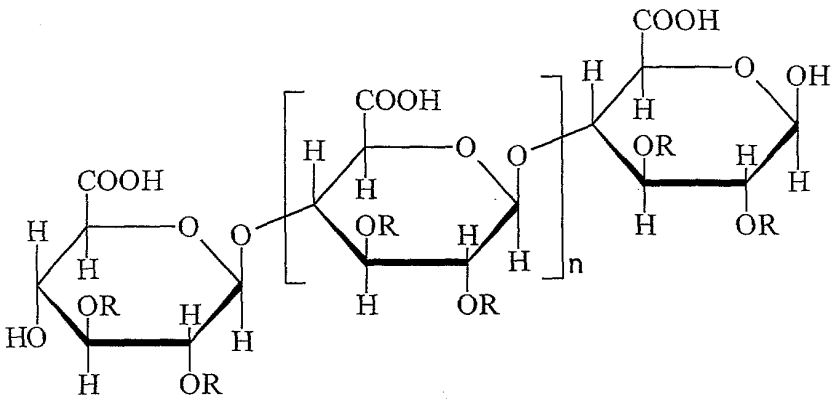
The mannuronan oligomer of DP4 (MAN) induces the strongest XET response (amplification in 20 minutes by a factor or 2.12); the glucuronan oligomer of DP8 (GLUC) and the hormone (GA3) are less effective (amplification in 20 minutes by a factor of 1.62 and 1.12 respectively). The reference XET activity is that of the non-elicited protoplasts (CONTROL).

Legend to figure 1: XET activity is indicated on the Y-axis, and time on the X-axis; the curve following the triangles corresponds to the results obtained with the mannuronan oligomer of DP4 (MAN), the curve following the crosses corresponds to the results obtained with the glucuronan oligomer of DP8 (GLUC), the curve following the circles corresponds to the results obtained with the hormone (GA3), the curve following the squares corresponds to the results obtained with the control.

CLAIMS

1. Use of compounds chosen from:

– the following 1,4 β-D-glucuronan polymers of formula (I):



in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$.

– and/or the β(1-4) chain glycuronic oligosaccharides derived from polymers of formula (I), and of which the number of saccharidic units is less than approximately 30,

– and/or the esters and/or ethers corresponding to polymers of formula (I) or to the above mentioned oligosaccharidic derivatives,

* as phytosanitary products within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase,
* and/or as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and/or the enzyme 1,4 β-D-glucanase, and/or xyloglucan endotransglycolase.

2. Use according to claim 1, of the compounds chosen from those mentioned in claim 1, as phytosanitary products within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, such as the protection of plants against pathogens, notably against bacteria, viruses, fungi, or the adaptation of the plants to an abiotic stress, in particular adaptation to cold, or to raised ozone levels.

3. Use according to claim 1 or 2 of 1,4 β-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

4. Use according to claim 1 or 2 of 1,4 β-D-glucuronan polymers of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$, the weight percentage of $COCH_3$ preferably being between 0 and 30.5.

5. Use according to claim 1 or 2 of β(1-4) chain glycuronic oligosaccharides, such as the oligo 1,4 β-D-glucuronans, the oligo 1,4 β-D-mannuronans, and the oligo 1,4 β-D-guluronans, whose DP is less than 30, and preferably between 2 and 15.

6. Use according to claim 5 of glycuronic oligosaccharides chosen from the following:

- the oligo 1,4 β-D-glucuronans of DP8, and of average DP 8
- the oligo 1,4 β-D-mannuronan of DP4,
- the oligo 1,4 β-D-guluronan of DP4.

7. Use according to claim 1 of the compounds chosen from those mentioned in claim 1, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and/or the enzyme 1,4 β-D-glucanase, and/or xyloglucan endotransglycolase.

8. Use according to claim 7 of the compounds chosen from those mentioned in claim 1, as biofertilizers within the framework of control of one or more stages of plant development, such as the control of fruit maturation, abscission, growth of the pistil or maturation of the anthers, and/or control of the organization of cell walls during expansion of the tissues, and/or to reinforce the plant cell walls and adapt them to environmental stimuli.

9. Use according to claim 7 or 8, of oligo 1,4 β-D-glucuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the

framework of uses linked to their activity of amplifying the enzyme 1,3 β-D-glucanase, and the enzyme 1,4 β-D-glucanase, within the framework of control of one or more stages of plant development, such as the control of fruit maturation, abscission, growth of the pistil or maturation of the anthers.

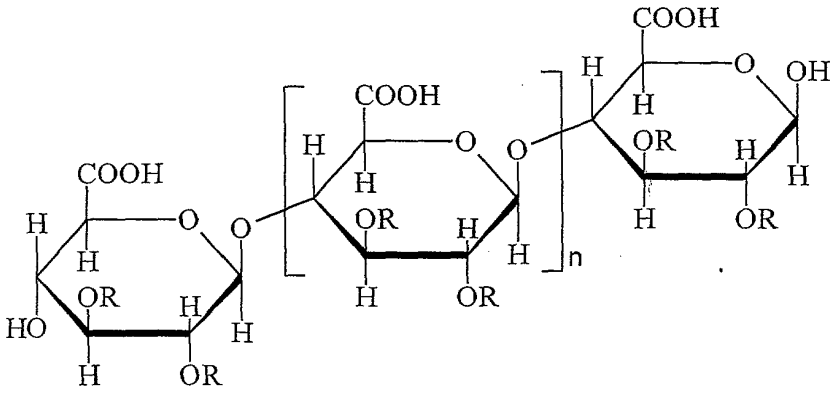
10. Use according to claim 9, of the oligo 1,4 β-D-glucuronan of average DP 8.

11. Use according to claim 7 or 8, of oligo 1,4 β-D-mannuronans, whose DP is below approximately 30, and preferably between 2 and 15, as biofertilizers within the framework of uses linked to their activity of amplifying the enzyme xyloglucan endotransglycolase within the framework of the control of organization of cell walls during expansion of the tissues and/or to reinforce the plant cell walls and adapt them to environmental stimuli.

12. Use according to claim 11, of the oligo 1,4 β-D-mannuronan of DP 4.

13. Phytosanitary products and/or biofertilizers characterized in that they include at least one compound chosen from:

– the following 1,4 β-D-glucuronan polymers of formula (I):



in which n is an integer between approximately 300 and approximately 2500, and R represents H or $COCH_3$,

– and/or the β(1-4) chain glycuronic oligosaccharides derived from polymers of formula (I), and of which the number of saccharidic units is less than approximately 30,

– and/or the esters and/or ethers corresponding to the polymers of formula (I) or to the above mentioned glycuronic oligosaccharidic derivatives.

14. Phytosanitary products according to claim 13, characterized in that they include at least one 1,4 β-D-glucuronan polymer of formula (I) in which n is an integer between approximately 300 and approximately 2500, and R represents H.

15. Phytosanitary products according to claim 13, characterized in that they include at least one β(1-4) chain glycuronan oligosaccharide, such as the oligo 1,4 β-D-glucuronans, the oligo 1,4 β-D-mannuronans, and the oligo 1,4 β-D-guluronans, whose DP is less than 20, and preferably between 5 and 15.

16. Phytosanitary products according to claim 15, characterized in that they include at least one glycuronic oligosaccharide chosen from the following:

– the oligo 1,4 β-D-glucuronans of DP8, and of average DP 8
– the oligo 1,4 β-D-mannuronan of DP4,
– the oligo 1,4 β-D-guluronan of DP4.

17. Biofertilizers according to claim 13, characterized in that they include at least at least one oligo 1,4 β-D-glucuronan, whose DP is less than approximately 30, and preferably between 2 and 15 such as the oligo 1,4 β-D-glucuronan of average DP 8.

18. Biofertilizers according to claim 11, characterized in that they include at least at least one oligo 1,4 β-D-mannuronan, whose DP is less than approximately 30, and preferably between 2 and 15, such as the oligo 1,4 β-D-mannuronan of DP 4.

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(71) Déposant *(pour tous les États désignés sauf US)*: **CENTRAL NATIONAL DE LA RECHERCHE SCIENTIFIQUE** [FR/FR]; 3, rue Michel-Ange, F-75794 Paris cedex 16 (FR).

(72) Inventeurs; et

(75) Inventeurs/Déposants *(pour US seulement)*: **LIENART, Yvette** [FR/FR]; Saint Nizier d'Uriage, F-38410 Uriage (FR). **HEYRAUD, Alain** [FR/FR]; 6, côte de Verdaret, F-38113 Veurey-Voroize (FR). **SEVENOU, Olivier** [FR/GB]; 1 Hight Street, Kegworth DE74 2DA (GB).

(74) Mandataires: **DEMACHY, Charles** etc.; Grosset-Fournier & Demachy SARL, 20, rue de Maubeuge, F-75009 Paris (FR).

(81) États désignés *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

(54) Title: USE OF GLYCURONIC POLYSACCHARIDES AND OLIGOSACCHARIDES AS PHYTOSANITARY PRODUCTS AND/OR FERTILISERS

(54) Titre: UTILISATION DE POLYSACCHARIDES ET D'OLIGOSACCHARIDES GLYCURONIQUES EN TANT QUE PRODUITS PHYTOSANITAIRES ET/OU FERTILISANTS

(57) Abstract: The invention concerns the use of compounds selected among 1,4 β-D-glucuronans, and/or glycuronic polysaccharides derived from polymers of formula (I), and whereof the number of saccharide units is less than about 30, and/or esters and/or ethers corresponding to polymers of formula (I) or said oligosccahride derivatives, as phytosanitary products in applications related to their activity for amplifying the 1,3 β-D-glucanase enzyme; and/or as biofertilisers in applications related to their activity amplifying the 1,3 β-D-glucanase enzyme, and/or the 1,4 β-D-glucanase, and/or the xyloglucan endotransglycolase.

(57) Abrégé: L'invention a pour objet l'utilisation de composés choisis parmi les polymères 1,4 β-D-glucuronanes, et/ou les oligosaccharides glycuroniques dérivés des polymères de formule (I), et dont le nombre d'unités saccharidiques est inférieur à environ 30, et/ou les esters et/ou éthers correspondants aux polymères de formule (I) ou aux dérivés oligosaccharidiques susmentionnés, en tant que produits phytosanitaires dans le cadre d'applications liées à leur activité d'amplification de l'enzyme 1,3 β-D-glucanase, et/ou en tant que biofertilisants dans le cadre d'applications liées à leur activité d'amplification de l'enzyme 1,3 β-D-glucanase, et/ou de l'enzyme 1,4 β-D-glucanase, et/ou de la xyloglucane endotransglycolase.

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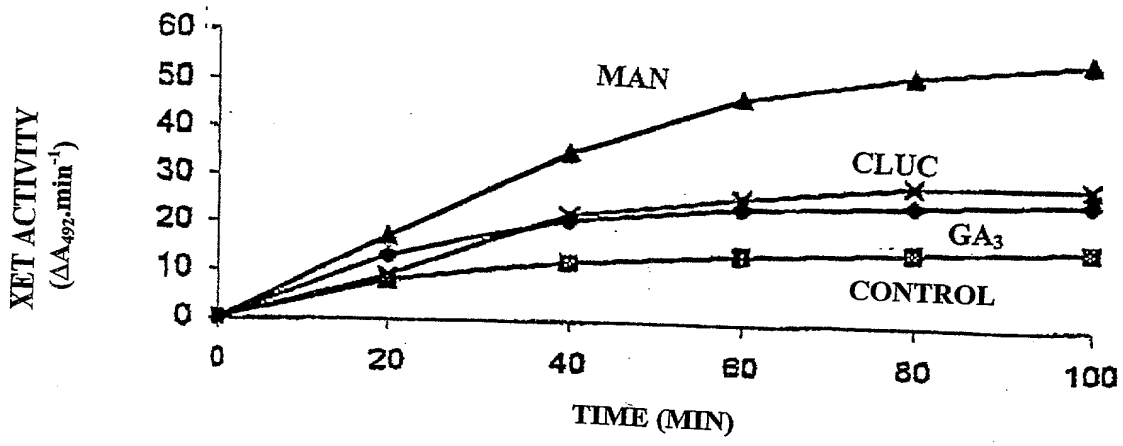
XET ACTIVITY
($\Delta A_{492}.min^{-1}$)
60
50
40
30
20
10
0
0
20
40
60
80
100
TIME (MIN)
MAN
CLUC
GA_3
CONTROL

FIGURE 1

61559

Attorney Docket No. USB 98 BC CNR PHY

COMBINED DECLARATION AND POWER OF ATTORNEY 61559/Nevalt

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: USE OF GLYCURONIC POLYSACCHARIDES AND OLIGOSACCHARIDES AS PHYTOSANITARY PRODUCTS AND/OR FERTILISER

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

☐ is attached hereto.
☐ was filed on _____ as application Serial No. _____ and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

☒ was described and claimed in International application No. PCT/FR00/01761 filed on June 23, 2000 and as amended on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
FR	99/08135	25-06-1999	Yes

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below:

_____

Application No. Filing Date (Status—patented, pending, abandoned)

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

_____

(Application No.) (Filing Date) (Status--patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from **GROSSET-FOURNIER & DEMACHY** as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the registered patent attorneys represented by Customer No. 000466 to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, including: **Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Thomas W. PERKINS, Reg. No. 33,027, Roland E. LONG, Jr., Reg. No. 41,949, and Eric JENSEN, Reg. No. 37,855,**

c/o YOUNG & THOMPSON,
Second Floor,
745 South 23rd Street,
Arlington, Virginia 22202.

Address all telephone calls to Young & Thompson at 703/521-2297. Telefax: 703/685-0573.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: Yvette LIENART

Inventor's signature: ______ Date: 29 janvier 2002

Residence: Uriage, France Citizenship: France

Post Office Address: Saint Nizier d'Uriage, F-38410 Uriage, France
473 Route de CHAMP RUTI

Full name of second joint inventor, if any: Alain HEYRAUD

Inventor's signature: ______ 29 Date: 29 janvier 2002

Residence: Veurey-Voroize, France Citizenship: France

Post Office Address: 6, Cote du Verdaret, F-38113 Veurey-Voroize, France

Full name of third joint inventor, if any: Olivier SEVENOU

Inventor's signature: ______ Date: 31 Janvier 2002

Residence: Kegworth, United Kingdom Citizenship: France

Post Office Address: 1 Hight Street, GB-Kegworth DE74 2DA, United Kingdom
FLAT 5